Environmental Health Perspectives
Vol. 15, pp. 13-25, 1976

Structural and Functional Changes in Acute Liver Injury

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Carbon tetrachloride produces liver cell injury in a variety of animal species. The first structurally recognizable changes occur in the endoplasmic reticulum, with alteration in ribosome-membrane interactions. Later there is an increase in intracellular fat, and the formation of tangled nets of the ergastoplasm. At no time are there changes in mitochondria or single membrane limited bodies in cells with intact plasmalemma, although a relative increase in cell sap may appear. In dead cells (those with plasmalemma discontinuties) crystalline deposits of calcium phosphate may be noted. Functional changes are related to the endoplasmic reticulum and the plasma membrane. An early decrease in protein synthesis takes place; an accumulation of neutral lipid is related to this change. Later alterations in the ergastoplasmic functions (e.g., mixed function oxidation) occurs. Carbon tetrachloride is not the active agent; rather, a product of its metabolism, probably the $\cdot CCl_3$ free radical, is. The mechanisms of injury include macromolecular adduction and peroxide propagation. A third possibility includes a cascade effect with the production of secondary and tertiary products, also toxic in nature, with the ability to produce more widespread damage to intracellular structures.

Introduction

Species and individual survival is influenced by physical and biological environmental factors; the former include temperature, water availability, light, and oxygen; the latter biological agents may be hazardous in themselves or capable of producing poisons. Man's interaction with his environment is one of adaptation. He is influenced by his surroundings and in turn modifies them. As an example of our ingenuity and ability to adapt, note our survival in arctic circumstances when indeed we are tropical animals. Unfortunately, the results of this ingenuity burden the environment with additional products, some of which are injurious to the flora and fauna.

Man's recorded history includes numerous references to injury derived from his modification of the environment. These include biblical suggestions of poisoning, the murder of Socrates using hemlock, the production of the "soot wart" in chimney sweeps, and, more recently, the contamination of our environment by petrochemical derivatives. Of the several goals of the toxicologist and the experimental pathologist, the identification of the mechanisms of action of these toxic agents—and thereby the gaining of an understanding of the disease process produced—stands out. The information to be won has heuristic value in that it may be used to prevent disease or suggest therapeutic means of modifying the course of illness produced.

The number and variety of toxic agents are protean; the accumulated knowledge concerning their mechanisms of action is considerably smaller. It is embarrassing to admit that, notwithstanding our current degree of biological sophistication, there is not one toxin for which the entire spectrum of physiological response has a sound and firm scientific explanation. Of the several agents that have been investigated, halogenated hydrocarbons, ethionine, nitrosamines, and several of the plant alkaloids are unique in that a wealth of information is available concerning their actions and attendant cellular re-

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sponses. This review will deal briefly with halogenated hydrocarbons, specifically with chloroform and carbon tetrachloride (*1,2*).

Structural Changes in Carbon Tetrachloride Poisoning

From a historical standpoint, the investigations into the mechanisms of action of halogenated hydrocarbons on liver and kidney have been an out-growth of tissue changes seen in humans. Carbon tetrachloride was used as an anesthetic agent until it became apparent that the fulminant liver disease that followed its employ was related to the agent itself. Nonetheless, carbon tetrachloride had continued to be used as an antihelminthic until relatively recent times and may still be so employed in some areas (e.g., in sheep herds in Italy). Human consumption is usually accidental; if deliberate, consumption is frequently an instrument of suicide. Chloroform, which enjoyed an even greater reputation as an anesthetic agent, also decreased in popularity when it became apparent that significant liver injury was a consistent side effect. Chloroform persists in many pharmaceutical products, including nonprescription items available to the unwary. A popular patented cough medicine containing 0.5% chloroform is sold in many drug stores.

Descriptions of the effects of carbon tetrachloride and chloroform on mammalian organisms, including humans, were sporadic until in the late 1930s. At the University of London, Cameron and co-workers did detailed and critical light microscopy studies of the morphological effects of acute and chronic carbon tetrachloride poisoning of rats (*3*).

The changes that occur in animals exposed to carbon tetrachloride are rather uniform and independent of species. Similar (if not identical) changes occur in man, monkeys, guinea pigs, and rats (*4*). The time dependence and extent of the change are related to the route of administration, the dose to which the animal was exposed, and the previous history of the animal. Oral administration of carbon tetrachloride is associated wtith rapid absorption from the gut and, because of the hemodynamics of venous return, a rapid perfusion of the liver. Studies carried out by Recknagel and Littera (*5*) show clearly that during the first 90 min following oral administration, carbon tetrachloride had been absorbed into the blood stream, has passed through the liver, and is already widely distributed throughout the body. Following a nonlethal dose, morphological changes occur in the liver, kidney, and pulmonary alveolus. This discussion will be restricted to pathology of the liver only.

Within the first several hours the liver shows cell swelling, fatty deposition, and subtle cytoplasmic tinctorial shifts (Fig. 1). The cells in the central zone demonstrate a dispersion of the ribosomes from the surface of the endoplasmic reticulum and a loss of ribosome-ribosome interaction, both on these membranes and in the cell sap. In some of the cells toward the midzone a swelling and vesiculation of the endoplasmic reticulum occurs (Fig. 2). The cells in the periportal zone show varying degrees of ribosomal disaggregation (Fig. 3). It appears that these major changes are in the 70–80% of the lobule surrounding the central vein. Increased levels of exposure increase the extent of the injury, and cause cells more and more distant from the central vein to become affected.

It should be emphasized that critical electron micrograph analysis during the initial 3 hr following administration of carbon tetrachloride shows significant alternations only in those cell structures in or association with the ergastoplasm or ribosomes (*6-9*). Specifically, there is neither modification or chromatin distribution nor modification of mitochondrial morphology. Neither are there lysosomal or microbody changes; nor are changes in cell surface membranes apparent.

In the later 3–6 hr period, dispersal of the ribosomes in the central zone persists, there is an increased swelling of the endoplasmic reticulum in the midzone (Fig. 4), and the tangled nets of endoplasmic reticulum, designated "tubular aggregates" by Reynolds (*9*) appear (Fig. 5). From 6 to 12 hr, dissolution of the "dead" cells in the central zone occurs, as indicated by disruptions in the plasma membrane, clumping of nuclear material, and morphological alterations in mitochondria, including mitochondrial matrix swelling and the deposition of crystallinelike material. The cells in the periporal area do not share in these changes and show only ribosomal disaggregation, as noted above; in fact, by 12 hr re-formation of ribosomal aggregates in the periporal area can be noted. From 12 to 24 hr those cells with morphological change consistent with cell death literally "fall apart" (Figs. 6 and 7). The remaining cells show re-formation of ribosomal aggregates and increased nucleolar size. From 24 hr on, these cells

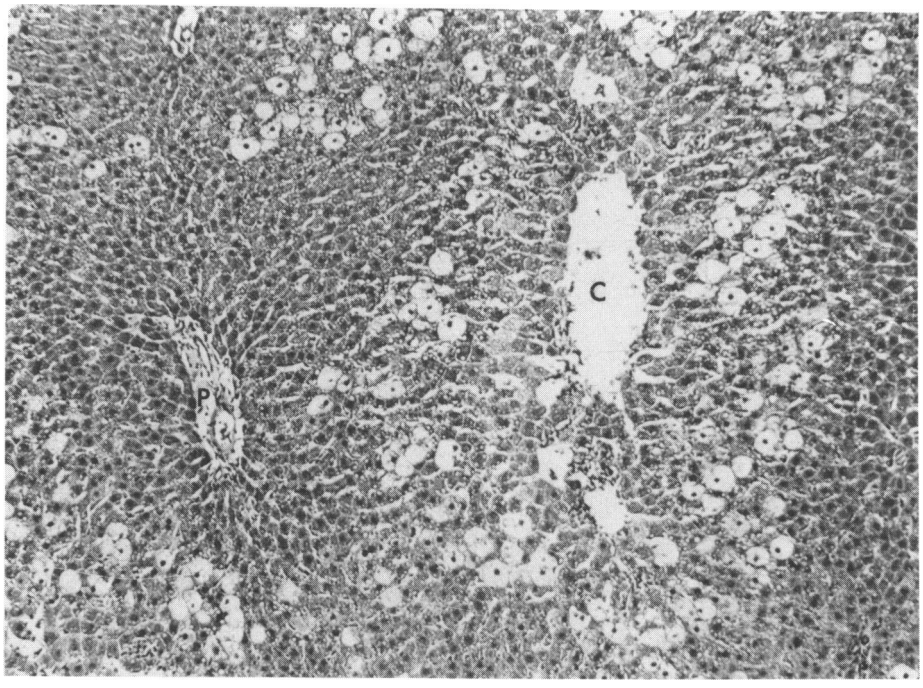

FIGURE 1. Light micrograph of rat liver 12 hr following oral administration of 0.25 ml of carbon tetrachloride/100 g body weight. The zonal nature of the cell injury produced by carbon tetrachloride is clearly demonstrated. The cells surrounding the central vein (C) show minimal morphological alteration but distinct tinctorial differences compared to those in the periportal area surrounding the portal triad (P). The nuclear morphology of these central zone cells is distorted and, in spite of the minimal disturbance, these cells are already dead. In the midzone a ring of dilated cells with distinct central nuclei are present. Those cells in the periportal area only show minimal change and will repopulate the lobules. This variance in appearance suggests an acute and overwhelming injury may provide little opportunity for the early appearance of distorted morphology, whereas a nonlethal injury (midzone) permits the cells to function in abnormal ways and shows more pronounced early structural changes. The specimen has been stained with hematoxylin and eosin. Magnification 500×. Reproduced from Smuckler and Arcasoy (4).

re-form the lobules; cell division is readily apparent. It should be pointed out that during this entire time period there is no significant morphological alteration either within the major bile ducts or in most of the Kupffer cells.

Functional Changes in Carbon Tetrachloride Poisoning

It was and still is hoped that the analysis of the functional changes that accompany carbon tetrachloride injury will allow the development of an understanding of the process of cell injury and cell death and provide correlation of these processes with changes described at the ultrastructural level. For several reasons it is instructive to review some of the earlier findings before correlating functional changes with the structural changes seen with the electron microscope.

Fatty Change

The accumulation of stainable lipid in cells is a feature believed to represent injury. Historically, two possible mechanisms of lipid accumulation were proposed: infiltration; the transport of fat from peripheral stores into the liver cell; and phanerosis; transformation of proteins and lipids within the cells into a histologically recognizable lipid form. The controversy concerning which mechanism was responsible for the observed changes waxed and waned for years until the turn of the century when experiments utilizing phosphorus poisoning indicated that the fat arose from the peripheral stores. These experiments were done in a very simple manner. The animals (in this case, dogs) were starved to deplete body stores of adipose tissue. They were then fed a lipid, either sheep tallow or linseed oil, whose iodine numbers and melting points were different from those of dog fat. The dogs were poisoned

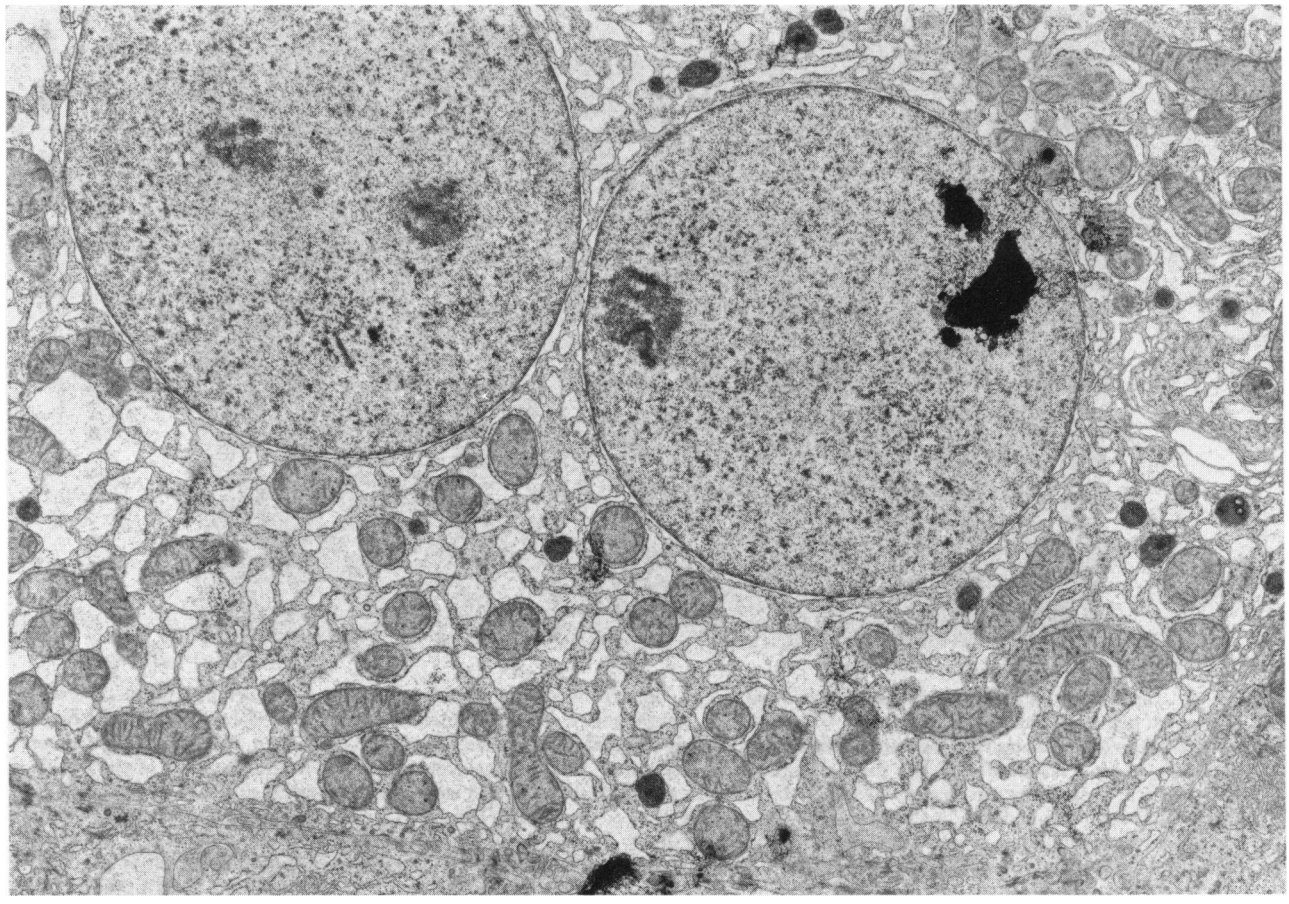

FIGURE 2. Electron micrograph of liver cells from the midzone 2 hr following carbon tetrachloride administration. The most significant feature is the dilated, vesiculated endoplasmic reticulum. This particular binucleate cell shows preservation of the mitochondria and other single membrane-limited bodies. Note also that there is a dispersion of the ribosomes from the ergastoplasmic membrane surface, as well as a loss of the ribosome-ribosome interaction within the cell sap. Approximate magnification 3500×.

when peripheral stores had been replenished. The fat that had accumulated in the liver was analyzed. It was found that the fat in the liver had iodine numbers consistent with those of newly formed peripheral stores (*10,11*). These data clearly suggested that synthesis of fat within the liver cell was not responsible for the lipid accumulation, but altered transport from peripheral stores to the liver or altered release (not recognized at the time) must have been responsible for its presence. More recently, tracer techniques have shown that indeed the source of the accumulated lipid is peripheral stores, but the mechanism underlying fat accumulation is a decrease in lipid secretion from liver cells, not an increase in transport to liver cells, (*12,13*). The basis for decreased secretion is discussed below.

Mitochondrial Changes

Cameron's early work (*13*) on the physiologic changes induced by carbon tetrachloride suggested that alteration in mitochondrial function might underlie the hepatocellular changes. Judah and co-workers (*14*) presented evidence that 24 hr following intoxication oxidative phosphorylation of mitochondria isolated from poisoned rat liver was altered, especially if the organelles were "aged" *in vitro*. The time lag between the structural distortion (6-12 hr) and the functional change (24 hr) is significant. The absence of altered mitochondrial morphology was puzzling, and more recent experiments could not identify respiratory disturbances in these organelles. It is now clear that the functional distortion of the

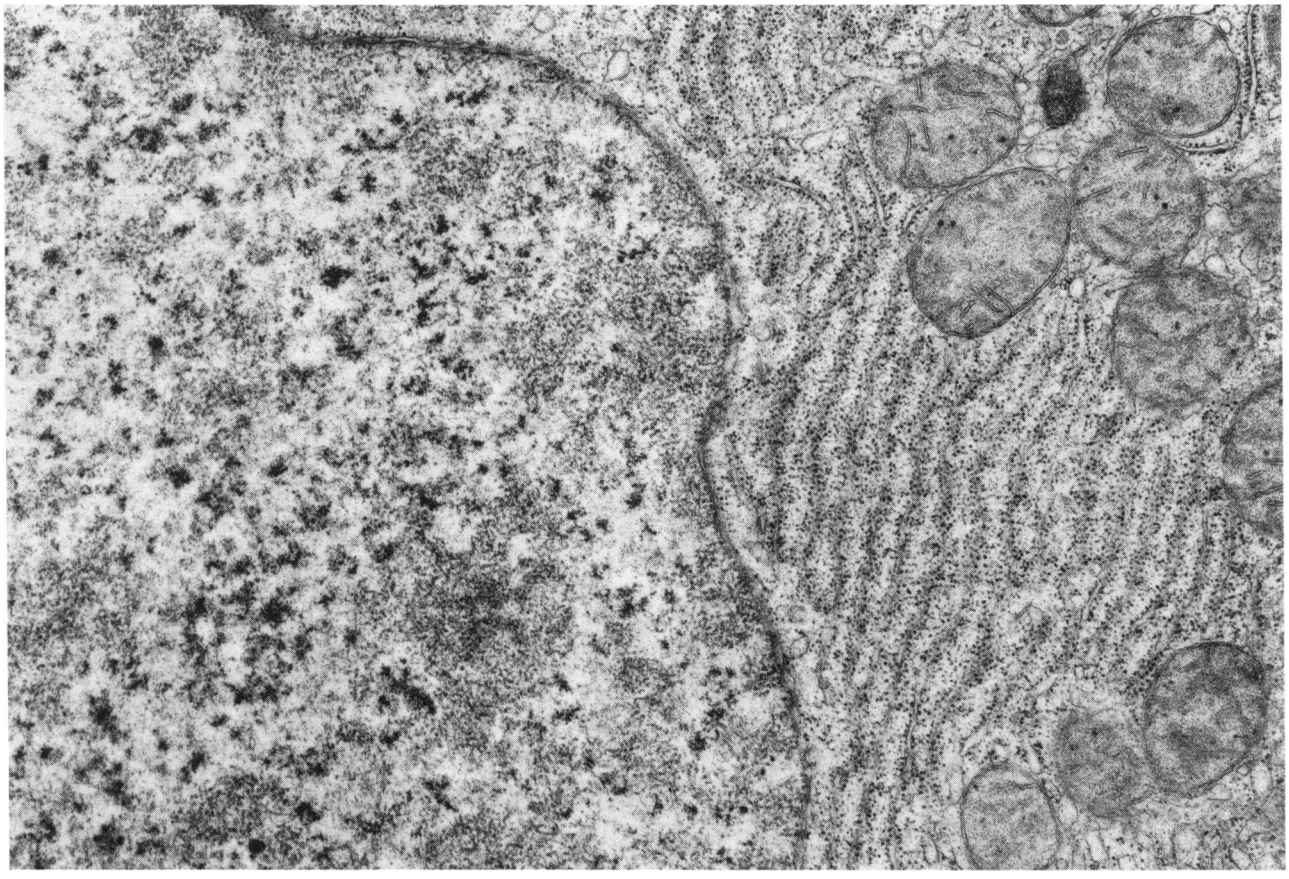

FIGURE 3. Micrograph taken from the midzone of a rat liver 1 hr following oral administration of carbon tetrachloride. It shows specifically the loss of ribosomal aggregates on the membrane surface as well as in the cell sap. In addition, there is a dispersal of the ribosomes from the membrane surface. Approximate magnification 30,000×. Reproduced from Smuckler and Arcasoy (*4*).

mitochondrion results from calcium accumulation. Dead cells in liver at 24 hr absorb large quantities of this ion. Homogenization places mitochondria in a calcium-enriched medium. The altered P/O ratios result from adventitious calcium uptake *in vitro*, not *in vivo* (*15*). Nonetheless, the experiments of Judah and coworkers represent a milestone, the introduction of quantitative functional biochemistry into the analysis of cell injury.

Functional Changes That Accompany Structural Modification

Protein Synthesis

Modification of rough endoplasmic reticulum and the loss of an ordered array of ribosomes in the cell sap suggested that defective protein synthesis might accompany these early structural effects of carbon tetrachloride poisoning (*8,15*). In fact, the incorporation of tracer amino acids into two proteins synthesized in the liver is reduced as early as two hours following carbon tetrachloride administration (Fig. 8). Subsequent studies have shown that synthesis not only of export proteins is reduced but of intracellular proteins as well. This correlates with structural changes in rough endoplasmic reticulum, where proteins for export are synthesized, and with the configuration of ribosomes in the cell sap, where intracellular proteins are made. Autoradiography has revealed that decreased leucine incorporation extends to periportal parenchymal cells (*17*). The observation that protein synthesis in Kupffer cells and other nonparencyhmal constituents is unaltered emphasizes the localization of altered protein synthesis to the hepatic parenchymal cell.

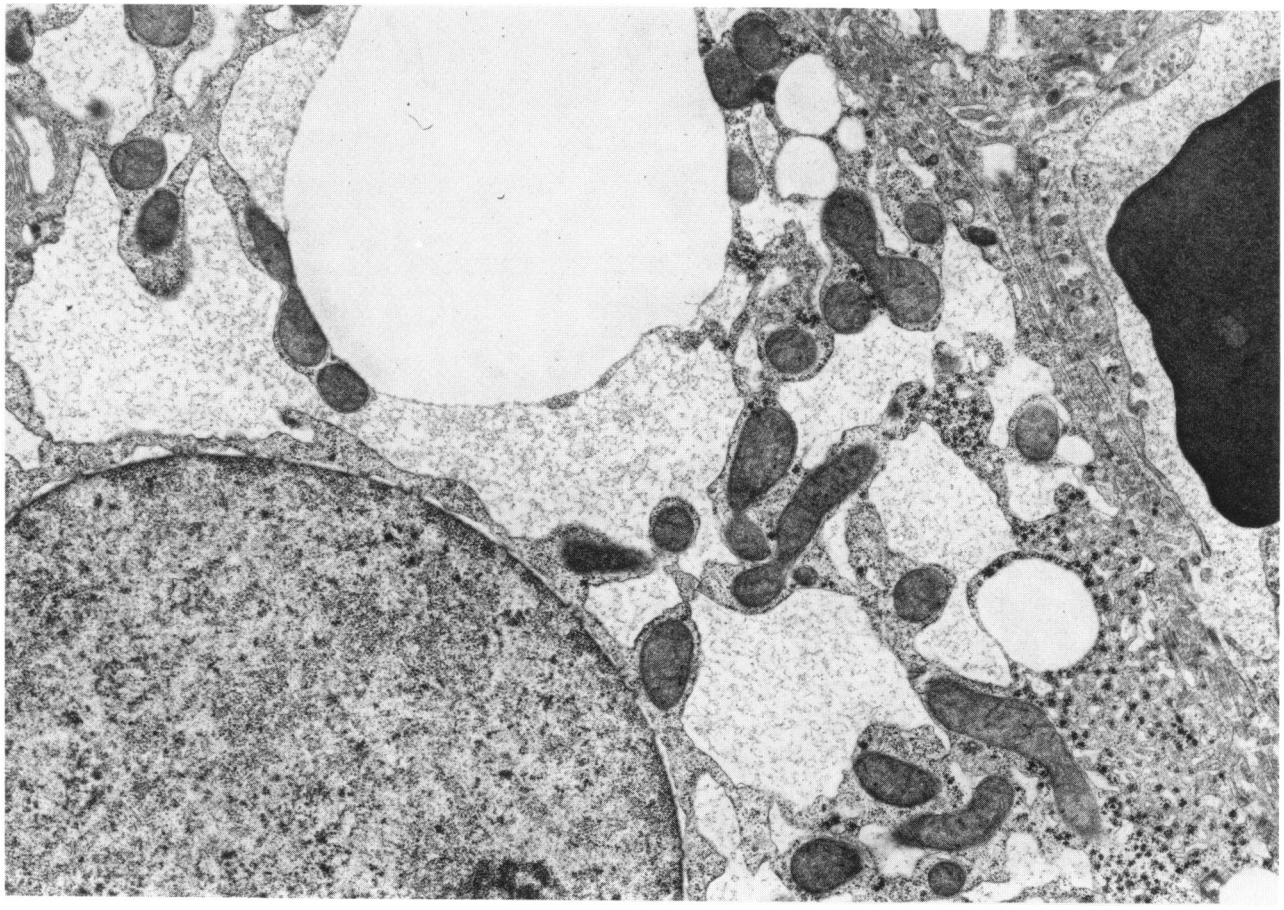

FIGURE 4. Micrograph showing the more marked dilation of the endoplasmic reticulum later in carbon tetrachloride injury, approximately 3 hr after administration. Note the granular material deposited within the cisternae of the endoplasmic reticulum and the large, clear space which contains (in life) lipid. This particular animal had not been fasted prior to intoxication, and glycogen deposits are present in the clumped endoplasmic reticulum in the upper part of the photograph. Approximate magnification 15,000×.

Cells that by morphologic criteria would be expected to die exhibit defects in protein synthesis, whereas those predicted to repopulate the liver seem relatively spared. This serves notice that there is a heterogeneous functional defect in these cells, but there is not a uniform morphological change.

The mechanism for the alteration of protein synthesis is not known. Studies of the ultrastructural and biochemical function of the nucleus have failed to reveal any defect in either RNA formation or transport (*18*). In fact, increased synthesis of DNA and RNA, which has been shown by autoradiography to be localized to the cells responsible for repair, takes place late in the course of intoxication, contemporaneous with regeneration and repair. If it assumed that the same mechanism obtains for both intra- and extra- cellular protein synthesis alterations, it follows that the ergastoplasmic membrane cannot be involved, and that therefore the defect resides in the ribsome-messenger complex. Ribosomes from animals intoxicated with carbon tetrachloride have been claimed to be "naked," "run off," or "deprogrammed". They can form polypeptides when given appropriate synthetic messenger and soluble factors, an observation that points to defective initiation or elongation.

One of the consequences of reduced protein synthesis is the altered secretion of neutral lipid from the liver cell alluded to above as a mechanism of fatty change. Lipid is transported to the liver cell as fatty acid bound to albumen, where it is absorbed from the plasma, and, within the liver

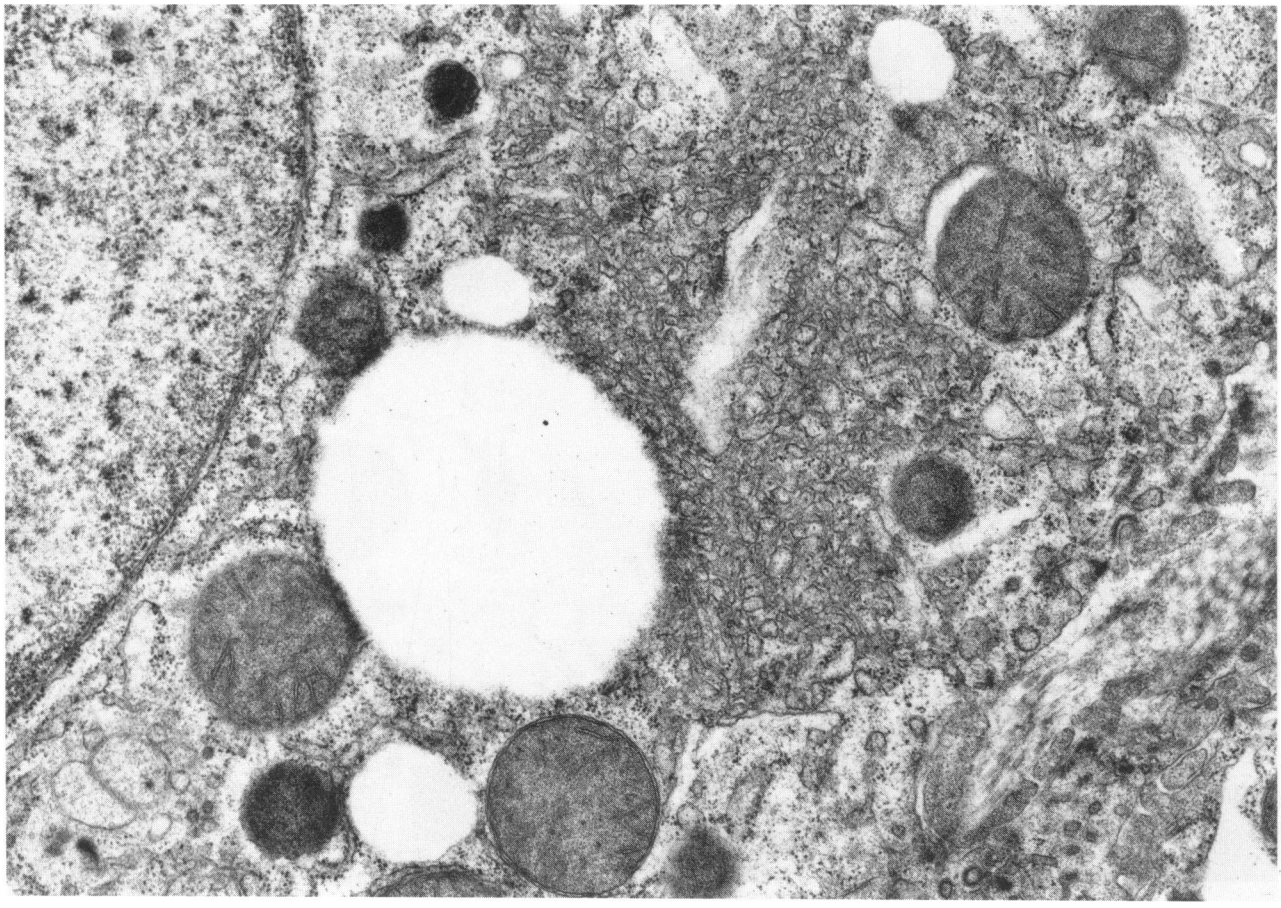

FIGURE 5. Micrograph of rat liver cell from the midzone taken 12 hr following carbon tetrachloride intoxication. Four lipid droplets (one large and three small) are present, and the large one is bordered on one side by a dense collection of the endoplasmic reticulum, termed "tubular aggregates." Approximate magnification 29,500×.

cell, esterified with glycerol to form neutral lipid. The excretion of neutral lipid requires a carrier whose synthesis is decreased as a consequence of reduced protein synthesis. The absence of carrier protein in part accounts for the accumulation of neutral lipid within the liver cell (*22–24*).*

Decreased protein synthesis has been hypothesized to be the basis of carbon tetrachloride toxicity. This, however, is certainly not the only mechanism operative in cell death or fatty change; ethionine poisoning is not associated with cell death, yet significant reduction of protein synthesis occurs (*26*). Cycloheximide inhibits protein synthesis in liver cells but fatty change does not result. The difficulty in determining whether decreased protein synthesis is a primary toxic effect or a secondary change highlights a major problem in the analysis of the mechanisms of injury, namely, the interaction of the toxin with the cell and with those several changes accompanying degeneration.

Intracellular Membrane Changes

Cell injury causes morphological and functional changes in the membraneous component of the endoplasmic reticulum. Structural changes include dilation and vesiculation of the membrane cisternae and formation of tangled webs of en-

* There are also data to suggest that the secretory process in the liver may be altered. The release process may involve cell membrane and ergastoplasmic interaction; it may also be energy dependent. As the accumulation of lipid occurs before significant reduction in ATP levels (*25*), the latter possibility seems less likely. Secretory changes and the reversible calcium accumulation (*vide infra*) suggest unidentified lesions in the plasmalemma.

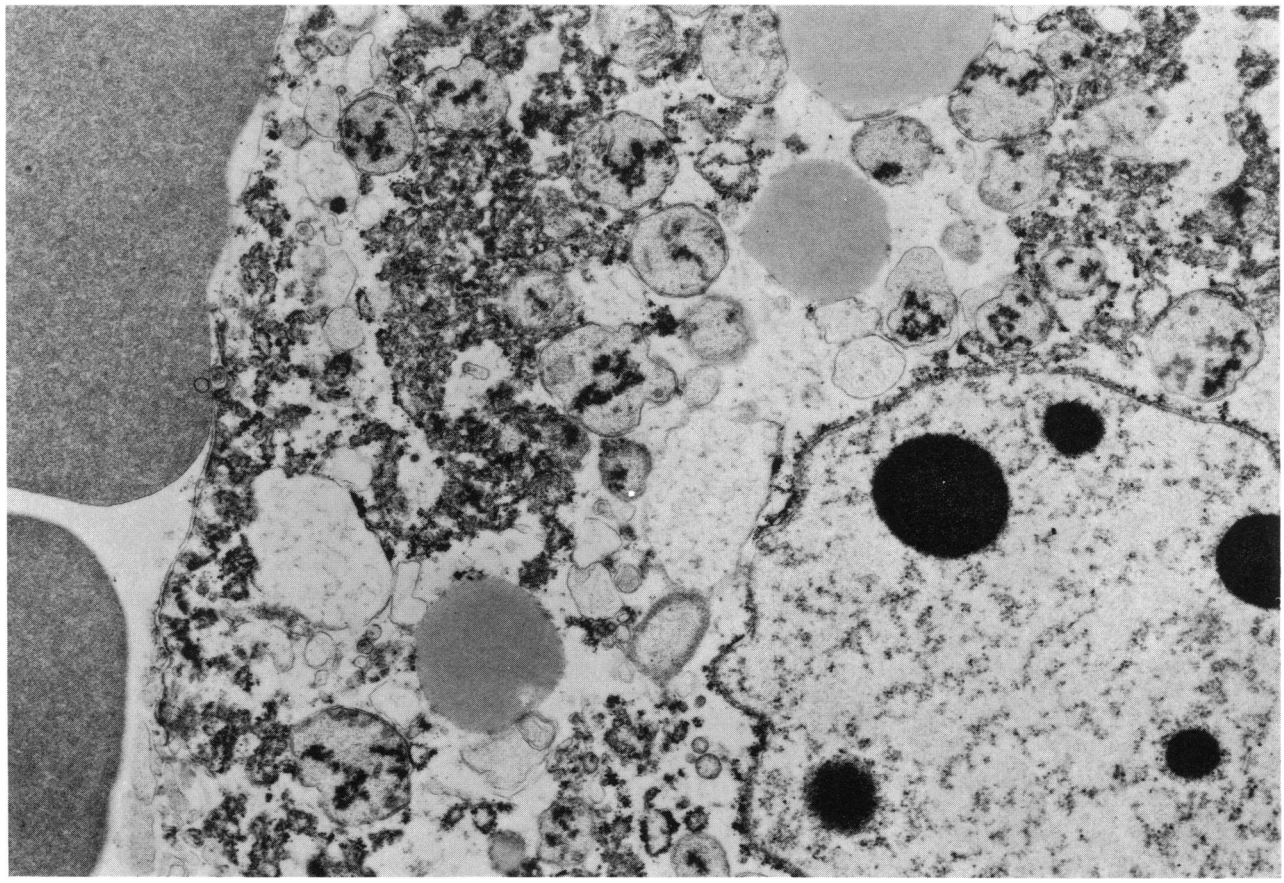

FIGURE 6. Micrograph of a centrally placed cell 24 hr following carbon tetrachloride intoxication. The nucleus demonstrates karyorrhexis; the endoplasmic reticulum is particularly clumped and shows the accretion of electron-dense material within the clumps. In addition, several lipid droplets are present, and the mitochondria show markedly altered morphology and the presence of calcium phosphate crystalloids within their matrix. Reproduced from Smuckler and Arcasoy (*4*). Magnification 20,060×.

doplasmic reticulum—Reynolds' tubular aggregates (see above). These tubular aggregates appear to be a degenerative modulation of the endoplasmic reticulum, which occurs concomitantly with altered membrane function. The one-to-one significance of this change is not known. Glucose-6-phosphatase activity is lost, and functional alterations occur in the microsomal electron transport chain (*21, 27–29*). More recent work has suggested that carbon tetrachloride is metabolized within the membranous system and that some metabolic product may be responsible for the structural and enzymic changes (*1*).

In those cells that survive the initial insult there is a rapid restoration of certain components of the endoplasmic reticulum, noticeably the return of cytochrome b_5 to control levels, which takes place long before the restoration of the remaining components of the endoplasmic reticulum (*30*). It has been argued that the reappearance of b_5 before repair of other components occurs suggests an altered phenotype following a single carbon tetrachloride exposure.

Plasmalemma Changes Associated with Death

The period of recovery is associated with two major processes; lethally injured cells in the central zone lyse, and lobules are re-formed by cells in the periportal zone.

Early in the intoxication (approximately 90 min), an accumulation and secondary secretion of calcium occurs in lethally injured cells, while ATP levels remain unaffected (*25, 31*). It is tempting to speculate that some transport mechanism has been altered early in the injury, as the

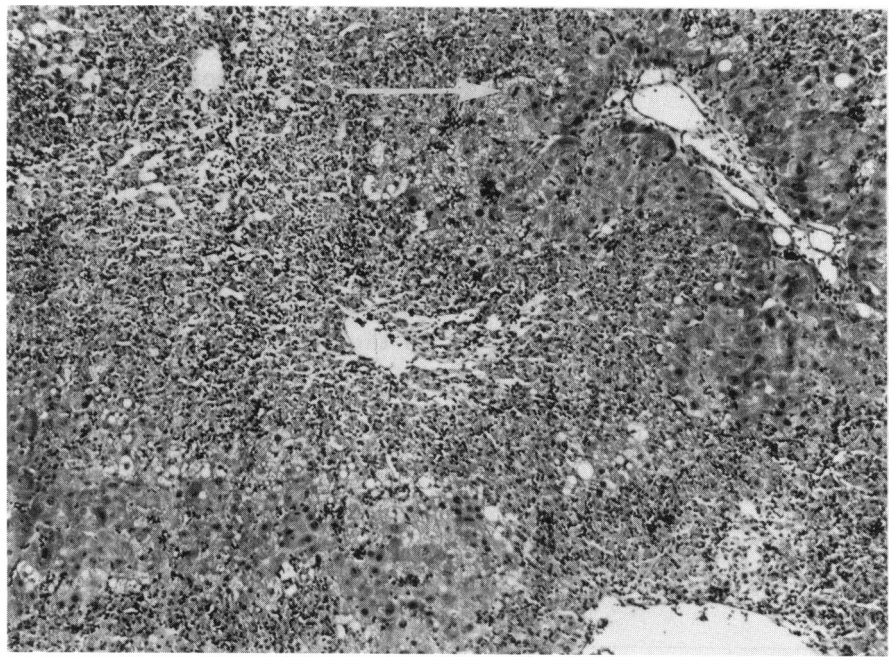

FIGURE 7. Section taken 48 hr following carbon tetrachloride injury, showing the demarcation of zonal necrosis and the very active regeneration in periportal cells. Magnification 500× Reproduced from Smuckler and Arcasoy (*4*).

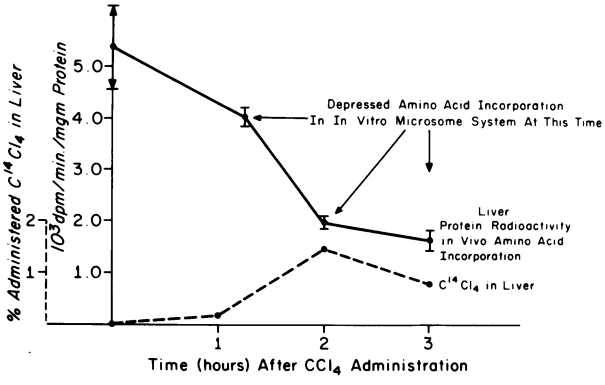

FIGURE 8. Relationship between the presence of carbon tetrachloride in the liver and the timepoints at which depressed proteins have been measured both *in vivo* and *in vitro*. It is readily apparent that in the first hour there is significant modulation of the capacity of the liver to make protein, yet the peak appearance of carbon tetrachloride has yet to reach the liver. Reproduced from Smuckler and Arcasoy (*4*).

calcium absorption occurs without any observable structural change in the plasmalemma or shift in transmembrane potential (*32*). By 3 hr, there is a return to normal cellular metal ion levels, indicating the absence of significant and permanent changes in plasmalemma function (Fig. 9). From 6 to 12 hr following intoxication, lethally injured cells in the central zone show the intracellular structural modification associated with dying. In addition, the transmembrane potential decreases slowly over time—but now with no apparent ion fluxes. When death occurs, the plasma membrane ruptures, allowing continuity between the inside and outside of the cell, and thereby the loss of any potential difference. Subsequent ionic shifts are governed by thermodynamics. It should be noted that until the actual rupture of the plasma membrane, which occurs with death, no structural change in the membrane can be detected.

Mechanisms of Action of Carbon Tetrachloride Injury

The evidence that carbon tetrachloride itself is not responsible for the changes seen in the liver is threefold: lack of synchrony between structural and functional changes; unresponsiveness of isolated organelles to direct addition of carbon tetrachloride, and correlation of the extent of injury with the level of drug metabolizing enzymes (*1*, *33-39*). The latter complex has been shown to dehalogenate the parent compound. The path-

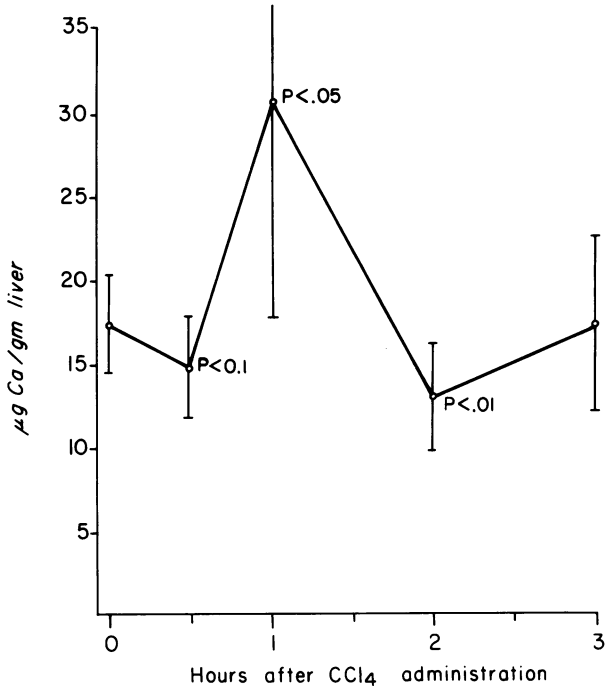

FIGURE 9. Graphic representation of the intracellular calcium level in liver following carbon tetrachloride administration. There is an initial lag followed by roughly a doubling of the intracellular calcium concentration which is followed very closely by a restitution of the normal calcium levels within the cell. Reproduced from Smuckler and Arcasoy (*4*).

ways of metabolism and intermediates formed from carbon tetrachloride are not all identified, but homolytic cleavage has been suggested as an initial step (*40*) and chloroform and carbon dioxide derived from carbon tetrachloride has been isolated (*42*). One of the metabolic products is suggested to be the proximate toxin. Two free-radical mechanisms for the action of the proximate toxin have been proposed: altered membrane function as a result of altered intracellular membrane structure; modified or decreased intracellular function as a result of free radical addition to cell macromolecules.

Both mechanisms involve an initial homolytic cleavage, yielding $\cdot CCl_3$. The first mechanism—which alters the lipids of intracellular membranes—is thought to be a chain reaction initiated by the action of $\cdot CCl_3$ on unsaturated fatty acids (*43–46*). The secondary lipid free radicals thus formed are postulated to propagate, and ultimately form peroxides. Lipid peroxides, or one of their breakdown products, malondialdehyde, were not detectable early in the course of injury (*47*). Malondialdehyde itself was shown to be metabolized, rates of formation and destruction being such that cell levels were too small for assay. Peroxides are detectable by diene conjugation early in the course of injury, but their concentration and the time dependence of their formation and disappearance are not easily related to the functional changes observed. This asynchrony points to a further complication, and secondary reaction products have been envisioned. Analysis of the structural changes in the ergastoplasmic membrane show a very restricted modification,

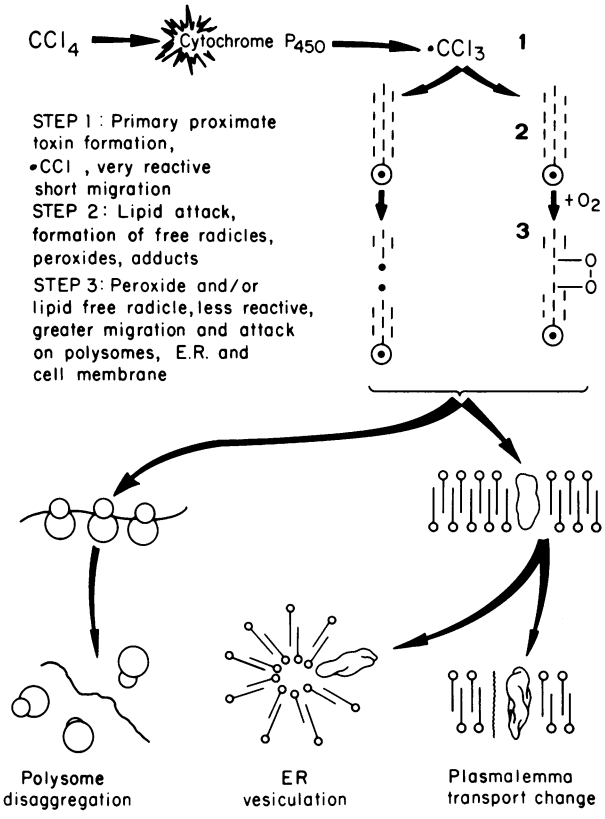

FIGURE 10. Hypothetical scheme for carbon tetrachloride-induced cell injury. Carbon tetrachloride is converted to a trichloromethyl free radical by cytochrome P450 and its associated cofactors (step 1). This radical is very reactive and is quenched by adjacent lipid and cytochromes after an apparent short migration (step 2), resulting in a discrete focus of destruction. The hypothetical lipid free radicals and peroxides formed are also reactive, but less so than $\cdot CCl_3$, allowing free-radical propagation to continue and further migration to take place. The peroxides and/or free radicals cause damage more remote than that caused by $\cdot CCl_3$. Either the peroxides, their breakdown products, lipid free radicals, or more remotely the $\cdot CCl_3$ radical react with polysomes causing their disaggregation and with both endoplasmic reticulum membranes and plasmalemma distorting functions and structure (step 3).

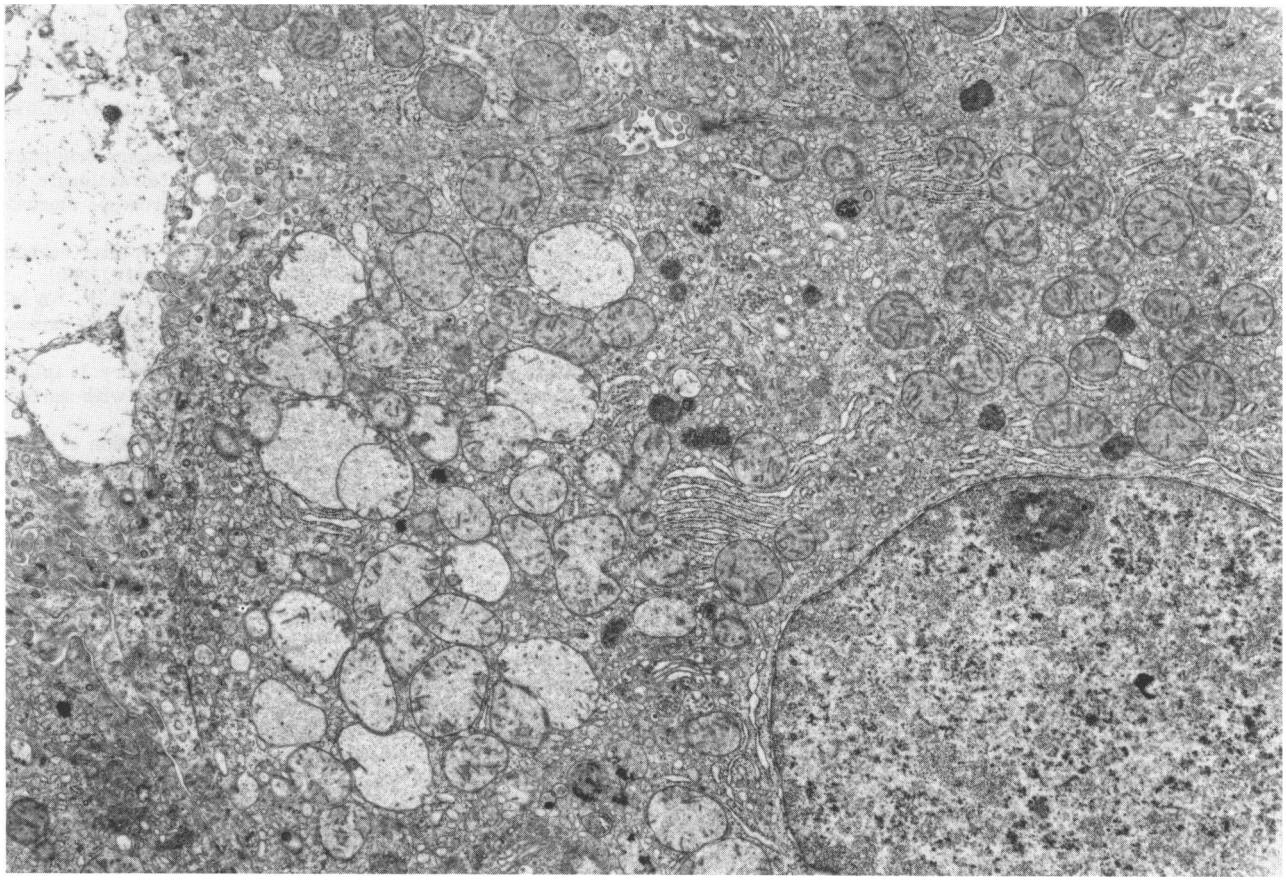

FIGURE 11. Micrograph illustrating some of the major problems in the interpretation of structural and functional changes. Within the one liver cell, 24 hr following carbon tetrachloride administration, structural changes within the mitochondria and single membrane-limited bodies appear in one cell region, while there is restitution of normal cellular architecture in the remainder. Near the sinusoidal surface, the endoplasmic reticulum is still clumped; a few liposomes remain within their cisternae, and the mitochondria are markedly distorted and swollen with shortened cristae. In the perinuclear area, however, the morphology of the cell has been restored to normal. Magnification 13,200×.

compatible with neither a free-radical attack nor a peroxide propagation (*48,49*). The basis for the paradox is not known.

The second mechanism suggests the formation of adducts between $\cdot CCl_3$ and cellular macromolecules (*49–53*). It also cannot be reconciled with all observed changes. The adducts formed are less random than can be accounted for by the known specificities of free radical adduct formation (*2, 49*). Adduction is also neither contemporaneous with nor sufficient in quantity to account for the observed changes.

Recently, Salter has directed attention to the reactivity and life span of the free-radical products. $\cdot CCl_3$ is particularly reactive and should therefore have a short radius of migration (*54*). If quenching of this proximate toxin occurs within a limited area of its formation, little damage will result. If, on the other hand, secondary products—for example, peroxides—with lower reactivity and therefore a potentially greater radius of action are formed, extensive and divergent disruption of cellular macromolecules can take place (Fig. 10). If carbon tetrachloride is assumed to be dehalogenated by cytochrome P-450 to give $\cdot CCl_3$ as the primary product, initial destruction of structure and function should be in the vicinity of the P-450 complex. This mechanism necessarily suggests a limited chemical modification, which is seen, and the potential for metabolic saturation, which also has been reported.

Secondary, less reactive products with the potential for a greater radius of action may be those responsible for the destruction of the mixed

function oxidase system and the alterations in protein synthesis. A cascade phenomenon may in fact be generated, higher order free radical reaction products causing damage to cell structures more and more remote from the initial lesion. Such a mechanism can indeed account for the widespread—but heterogeneous—destruction observed (Fig. 10).

Summary

To summarize, ultrastructural analyses demonstrated an early and pronounced change in the endoplasmic reticulum following carbon tetrachloride administration. Subsequent biochemical studies have shown that both ribosomal and membrane function are also modified. There is a decrease in both protein formation and in the capacity to undertake microsomal electron transport. The changes in anabolism correlate with the inability to transport lipid from the cell resulting in fatty degeneration, and with the decreased formation of essential cellular protein components. The actual mechanisms by which these changes occur, however, have escaped clearcut identification and, indeed, changes in the plasma membrane that can be detected functionally cannot be shown by ultrastructural techniques. Finally, several of the changes shown in dead cells would appear to be artifacts resulting from decreased cell function (e.g., loss of selective plasmalemma transport) and, although described in detail, their relevance to the process of cell death may be remote. These remaining problems demonstrate the necessity for combined structural and functional analyses and further point to the potential of combined approaches in unraveling the process of cell injury. Figure 11 indicates clearly the difficult task of unraveling these changes.

This work was supported in part by USPHS Grant AM 08686.

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